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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 9/16, A61K 38/46

A1

(11) International Publication Number: WO 00/09672

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/IB99/01484

(22) International Filing Date: 12 August 1999 (12.08.99)

(30) Priority Data:
60/096,374 13 August 1998 (13.08.98) US

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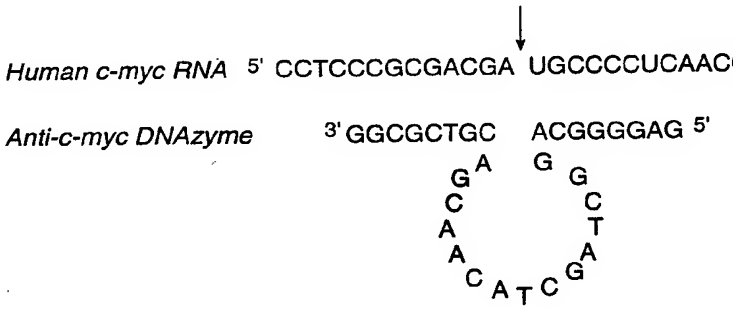
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNAZYMES AND METHODS FOR TREATING RESTENOSIS

(57) Abstract

This application provides a DNAzyme which specifically cleaves c–*myc* mRNA, comprising a 15–nucleotide catalytic domain and two binding domains, one binding domain contiguous with the 5' end of the catalytic domain and the other binding domain contiguous with the 3' end of the catalytic domain. This invention also provides a pharmaceutical composition for inhibiting the onset of restenosis, which comprises the instant DNAzyme and a pharmaceutically acceptable carrier suitable for topical administration. This invention further provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an angioplastic stent operably coated with a prophylactically effective dose of the instant pharmaceutical composition. Finally, this invention provides methods for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering either the instant pharmaceutical composition or angioplastic stent to the subject.



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DNAZYMES AND METHODS FOR TREATING RESTENOSIS

Field of the Invention

This invention relates to inhibiting the onset of restenosis using DNAzymes. The DNAzymes accomplish this end by cleaving mRNA encoding c-*myc*, whose expression in vascular smooth muscle cells is required for restenosis to occur.

Background of the Invention

Restenosis

Restenosis is a serious medical disorder which often occurs following angioplasty. This disorder afflicts 30%-60% of all angioplasty patients.

Restenosis is understood to be caused, at least in part, by excessive proliferation of smooth muscle cells ("SMC's") following vascular injury occurring during angioplasty. Several biological modulators are thought to facilitate this SMC proliferation. These modulators include platelet-derived growth factor ("PDGF"), fibroblast growth factor ("FGF") and insulin growth factor ("IGF") (Ross; Banscota; Libby; Gay). The induction of SMC proliferation by these modulators occurs via the intracellular transactivation of a number of important genes (Kindy; Gadeau). These genes include c-*myc*, c-*myb*, c-*fos* and PCNA (proliferating cell nuclear antigen), and generally are cell cycle-specific.

In particular, the c-*myc* gene is over-expressed in SMC's within 30 minutes to two hours of vascular trauma, and expression declines to normal levels within

12 hours thereafter. In other words, angioplasty causes vascular SMC injury, which triggers excess c-*myc* expression beginning 30 minutes to two hours after injury, and ending 12 hours after injury.

Restenosis is presently treated using radiation and pharmacological therapies. Radiation therapy includes either radioactive implants or delivery of a radioactive composition to the site being treated. Although radiation therapy has shown some promising results, the long-term side effects of intra-coronary radiation have yet to be established. Regarding pharmacological therapy, both the anti-thrombotin and anti-proliferation approaches employed to date are generally ineffective (Bennet).

DNAzymes

In human gene therapy, antisense nucleic acid technology has been one of the major tools of choice to inactivate genes whose expression causes disease and is thus undesirable. The anti-sense approach employs a nucleic acid molecule that is complementary to, and thereby hybridizes with, an mRNA molecule encoding an undesirable gene. Such hybridization leads to the inhibition of gene expression.

Anti-sense technology suffers from certain drawbacks. Anti-sense hybridization results in the formation of a DNA/target mRNA heteroduplex. This heteroduplex serves as a substrate for RNAse H-mediated degradation of the target mRNA component. Here, the DNA anti-sense molecule serves in a passive manner, in that it merely facilitates the required cleavage by endogenous RNAse H enzyme. This dependence on RNAse H confers limitations on the design of anti-sense

molecules regarding their chemistry and ability to form stable heteroduplexes with their target mRNA's. Anti-sense DNA molecules also suffer from problems associated with non-specific activity and, at higher concentrations, even toxicity.

As an alternative to anti-sense molecules, catalytic nucleic acid molecules have shown promise as therapeutic agents for suppressing gene expression, and are widely discussed in the literature (Haseloff; Breaker (1994); Koizumi; Otsuka; Kashani-Sabet; Raillard; and Carmi). Thus, unlike a conventional anti-sense molecule, a catalytic nucleic acid molecule functions by actually cleaving its target mRNA molecule instead of merely binding to it. Catalytic nucleic acid molecules can only cleave a target nucleic acid sequence if that target sequence meets certain minimum requirements. The target sequence must be complementary to the hybridizing regions of the catalytic nucleic acid, and the target must contain a specific sequence at the site of cleavage.

Catalytic RNA molecules ("ribozymes") are well documented (Haseloff; Symonds; and Sun), and have been shown to be capable of cleaving both RNA (Haseloff) and DNA (Raillard) molecules. Indeed, the development of in vitro selection and evolution techniques has made it possible to obtain novel ribozymes against a known substrate, using either random variants of a known ribozyme or random-sequence RNA as a starting point (Pan; Tsang; and Breaker (1994)).

Ribozymes, however, are highly susceptible to enzymatic hydrolysis within the cells where they are

intended to perform their function. This in turn limits their pharmaceutical applications.

Recently, a new class of catalytic molecules called "DNAzymes" was created (Breaker (1995); Santoro). DNAzymes are single-stranded, and cleave both RNA (Breaker (1994); Santoro) and DNA (Carmi). A general model for the DNAzyme has been proposed, and is known as the "10-23" model. DNAzymes following the "10-23" model, also referred to simply as "10-23 DNAzymes", have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. In vitro analyses show that this type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions under physiological conditions (Santoro).

DNAzymes show promise as therapeutic agents. However, DNAzyme success against a disease caused by the presence of a known mRNA molecule is not predictable. This unpredictability is due, in part, to two factors. First, certain mRNA secondary structures can impede a DNAzyme's ability to bind to and cleave its target mRNA. Second, the uptake of a DNAzyme by cells expressing the target mRNA may not be efficient enough to permit therapeutically meaningful results. For these reasons, merely knowing of a disease and its causative target mRNA sequence does not alone allow one to reasonably predict the therapeutic success of a DNAzyme against that target mRNA, absent an inventive step.

Summary of the Invention

This application provides a DNAzyme which specifically cleaves c-*myc* mRNA, comprising (a) a catalytic domain that has the nucleotide sequence GGCTAGCTACAACGA and cleaves mRNA at any purine:pyrimidine cleavage site at which it is directed, (b) a binding domain contiguous with the 5' end of the catalytic domain, and (c) another binding domain contiguous with the 3' end of the catalytic domain, wherein the binding domains are complementary to, and therefore hybridize with, the two regions immediately flanking the purine residue of the cleavage site within the c-*myc* mRNA, respectively, at which DNAzyme-catalyzed cleavage is desired, and wherein each binding domain is at least six nucleotides in length, and both binding domains have a combined total length of at least 14 nucleotides.

This invention also provides a pharmaceutical composition for inhibiting the onset of restenosis, which comprises the instant DNAzyme and a pharmaceutically acceptable carrier suitable for topical administration.

This invention further provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an agioplastic stent operably coated with a prophylactically effective dose of the instant pharmaceutical composition.

This invention still further provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a prophylactically effective dose of the

instant pharmaceutical composition to the subject at around the time of the angioplasty.

Finally, this invention provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering the instant angioplastic stent to the subject at the time of the angioplasty.

Brief Description of the Figures

Figure 1 shows the structure of a "10-23" DNAzyme described in Santoro. The cleavage site is indicated by an asterisk between X and Y. The substrate-binding domains are indicated by N's.

Figure 2 shows c-*myc* RNA-cleaving DNAzyme designs. The cleavage site for the c-*myc* DNAzyme was chosen at the AUG start codon of the human c-*myc* mRNA (2nd exon). Cleavage occurs between A and U as indicated.

Figure 3 shows the optimization of DNAzyme arm-length and chemical modification. C-*myc*-cleaving DNAzymes with different arm lengths were designed based on the "10-23" model. The 3'-3' terminal base inversion at the 3' end is indicated by a shadow **C** or **G** (3'INV).

Figure 4 shows the analysis of multiple turnover kinetics. Panel A shows a densitometric image, obtained using a PhosphorImager (Molecular Dynamics), of a 16% polyacrylamide gel, showing cleavage of synthetic c-*myc* mRNA under multiple turnover conditions. All reactions were performed with 200 pM DNAzyme and 2 nM, 4 nM, 8 nM, 16 nM, and 32 nM of substrate mRNA (as indicated). The incubation time for each reaction, ranging from 0-60 minutes, is indicated at the top of each lane. Panel B shows a plot of DNAzyme cleavage progress (nM) for each substrate concentration. These data were derived from densitometry measurements of cleaved bands shown in Panel A.

Figure 5 shows the in vitro cleavage of c-*myc* mRNA. 1.5 kb c-*myc* mRNA substrate were transcribed from a pGEM vector in the presence of ^{32}P-UTP. The cleavage

reaction was performed at 10 mM $MgCl_2$, 50 mM Tris.HCl, pH 7.5, 37°C for 60 minutes.

Figure 6 shows a stability assay of the 3'-inverted DNAzyme in human serum. DNAzymes were incubated with AB-type human serum (Sigma). Samples were collected at different time points as indicated, and labeled with ^{32}P. The labeled DNAzymes were analyzed on 16% PAGE gel. Typical gel patterns are shown here for unmodified (top right) and 3' inverted DNAzymes (bottom right).

Figure 7 shows the testing of c-*myc* mRNA-cleaving DNAzymes in SV-LT-SMC's. Growth-arrested SMC's were stimulated with 10% FBS-DME (Dulbecco's Modified Eagle Medium containing 0.5% fetal bovine serum) in the presence of 10 mM anti-c-*myc* mRNA DNAzyme designated Rs-6 (described below), 10 mM control oligonucleotide (same arm sequences as Rs-6, with an inverted catalytic core sequence), or liposome alone (DOTAP; i.e. N-[1-(2,3-dioleoyloxy)-N,N,N-trimethylammonium-methylsulfate). The data are displayed as mean ± SD.

Figure 8 shows dose-response experiments for Rs-6 DNAzyme in SMC's. The experimental details are as per Figure 7. The data are expressed as a percentage of the control.

Figure 9 shows c-*myc* expression in DNAzyme-treated SMC's. Cells were labeled with ^{35}S-methionine as described in Example 7, and immunoprecipitation was performed to determine the expression level of c-*myc* protein in DNAzyme-treated SMC's.

Figure 10 shows the genomic DNA sequence of the human c-*myc* gene (exons 1 and 2).

Detailed Description of the Invention

This invention is directed to inhibiting the onset of restenosis using DNAzyme technology. The disorder's onset, triggered by physical trauma to arterial smooth muscle during angioplasty, is characterized by a several-hour period of c-*myc* over-expression following shortly thereafter. This c-*myc* over-expression leads to excess SMC proliferation, and inhibition of this overexpression in turn inhibits the onset of restenosis. This invention exploits this "window of opportunity" of c-*myc* over-expression by applying a c-*myc* mRNA-specific DNAzyme to the area of trauma around the time of angioplasty, thereby cleaving the mRNA and inhibiting restenosis onset.

More specifically, this application provides a DNAzyme which specifically cleaves c-*myc* mRNA, comprising (a) a catalytic domain that has the nucleotide sequence GGCTAGCTACAACGA and cleaves mRNA at any purine:pyrimidine cleavage site at which it is directed, (b) a binding domain contiguous with the 5' end of the catalytic domain, and (c) another binding domain contiguous with the 3' end of the catalytic domain, wherein the binding domains are complementary to, and therefore hybridize with, the two regions immediately flanking the purine residue of the cleavage site within the c-*myc* mRNA, respectively, at which DNAzyme-catalyzed cleavage is desired, and wherein each binding domain is at least six nucleotides in length, and both binding domains have a combined total length of at least 14 nucleotides.

As used herein, "DNAzyme" means a DNA molecule that specifically recognizes and cleaves a distinct target nucleic acid sequence, which can be either DNA or RNA.

The instant DNAzyme cleaves RNA molecules, and is of the "10-23" model, as shown in Figure 1, named so for historical reasons. This type of DNAzyme is described in Santoro. The RNA target sequence requirement for the 10-23 DNAzyme is any RNA sequence consisting of NNNNNNNR*YNNNNNN, NNNNNNNNR*YNNNNN or NNNNNNR*YNNNNNNN, where R*Y is the cleavage site, R is A or G, Y is U or C, and N is any of G, U, C, or A.

Within the parameters of this invention, the binding domain lengths (also referred to herein as "arm lengths") can be of any permutation, and can be the same or different. Various permutations such as 7+7, 8+8 and 9+9 are envisioned, and are exemplified more fully in the Examples that follow. It is well established that the greater the binding domain length, the more tightly it will bind to its complementary mRNA sequence. According, in the preferred embodiment, each binding domain is nine nucleotides in length. In one embodiment, the instant DNAzyme has the sequence TGAGGGGCAGGCTAGCTACAACGACGTCGTGAC (also referred to herein as "Rs-6").

In applying DNAzyme-based treatments, it is important that the DNAzymes be as stable as possible against degradation in the intra-cellular milieu. One means of accomplishing this is by incorporating a 3'-3' inversion at one or more termini of the DNAzyme. More specifically, a 3'-3' inversion (also referred to herein simply as an "inversion") means the covalent phosphate bonding between the 3' carbons of the terminal nucleotide and its adjacent nucleotide. This type of bonding is opposed to the normal phosphate bonding between the 3' and 5' carbons of adjacent nucleotides, hence the term "inversion." Accordingly, in the preferred embodiment, the 3'-end nucleotide

residue is inverted in the binding domain contiguous with the 3′ end of the catalytic domain. In addition to inversions, the instant DNAzymes can contain modified nucleotides. Modified nucleotides include, for example, N3′-P5′ phosphoramidate linkages, and peptide-nucleic acid linkages. These are well known in the art (Wagner).

In this invention, any contiguous purine: pyrimidine nucleotide pair within the c-*myc* mRNA can serve as a cleavage site. In the preferred embodiment, purine:uracil is the desired purine:pyrimidine cleavage site.

The c-*myc* mRNA region containing the cleavage site can be any region. For example, the location within the c-*myc* mRNA at which DNAzyme-catalyzed cleavage is desired can be the translation initiation site, a splice recognition site, the 5′ untranslated region, and the 3′ untranslated region. In one embodiment, the cleavage site is located at the translation initiation site.

The sequences of human c-*myc* mRNA, and/or DNA encoding same, are well known (Bernard). As used herein, "c-*myc* mRNA" means any mRNA sequence encoded by the human c-*myc* DNA sequence shown in Figure 10 or by any naturally occurring polymorphism thereof. C-*myc* mRNA includes both mature and immature mRNA. Within the parameters of this invention, determining the c-*myc* mRNA cleavage site, the required sequences of each binding region, and thus the sequence of then entire DNAzyme, can be done according to well known methods.

This invention also provides a pharmaceutical composition for inhibiting the onset of restenosis,

which comprises the instant DNAzyme and a pharmaceutically acceptable carrier suitable for topical administration.

In this invention, topically administering the instant pharmaceutical composition can be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The topical administration can be performed, for example, via catheter and topical injection, and via coated stent as discussed below.

Pharmaceutical carriers for topical administration are well known in the art, as are methods for combining same with active agents to be delivered. The following delivery systems, which employ a number of routinely used carriers, are only representative of the many embodiments envisioned for administering the instant composition.

Topical delivery systems include, for example, gels and solutions, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In the preferred embodiment, the pharmaceutically acceptable carrier is a liposome or a biodegradable polymer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N^{I},N^{II},N^{III}-tetramethyl-N,N^{I},N^{II},N^{III}-tetrapalmitylspermine and dioleoyl phosphatidyl-ethanolamine (DOPE)(GIBCO BRL); (2) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-trimethyl-ammoniummethylsulfate) (Boehringer

Manheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

This invention further provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an agioplastic stent operably coated with a prophylactically effective dose of the instant pharmaceutical composition.

Angioplastic stents, also known by other terms such as "intravascular stents" or simply "stents", are well known in the art. They are routinely used to prevent vascular closure due to physical anomalies such as unwanted inward growth of vascular tissue due to surgical trauma. They often have a tubular, expanding lattice-type structure appropriate for their function, and can optionally be biodegradable.

In this invention, the stent can be operably coated with the instant pharmaceutical composition using any suitable means known in the art. Here, "operably coating" a stent means coating it in a way that permits the timely release of the pharmaceutical composition into the surrounding tissue to be treated once the coated stent is administered. Such coating methods, for example, can use the polymer polypyrrole. Stents, and methods and compositions for coating same, are discussed in detail in U.S. Serial No. 60/091,217.

Determining a prophylactically effective dose of the instant pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the prophylactically effective dose contains between about 0.1 mg and about 1 g of the instant DNAzyme. In another embodiment, the

prophylactically effective dose contains between about 1 mg and about 100 mg of the instant DNAzyme. In a further embodiment, the prophylactically effective dose contains between about 10 mg and about 50 mg of the instant DNAzyme. In yet a further embodiment, the prophylactically effective dose contains about 25 mg of the instant DNAzyme.

This invention further provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a prophylactically effective dose of the instant pharmaceutical composition to the subject at around the time of the angioplasty. As used herein, administering the instant pharmaceutical composition "at around" the time of angioplasy can be performed during the procedure, or immediately before or after the procedure. The administering can be performed according to known methods such as catheter delivery. "Inhibiting" the onset of restenosis means either lessening the severity of restenosis which occurs after angioplasty, or preventing the onset of restenosis entirely. In the preferred embodiment, inhibiting the onset of restenosis means preventing the onset of restenosis entirely.

Finally, this invention provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering the instant angioplastic stent to the subject at the time of the angioplasty.

This invention will be better understood by reference to the Examples which follow, but those skilled in the art will readily appreciate that they are only illustrative of the invention as described more fully in

the claims which follow thereafter. In addition, various documents are cited throughout this application. The disclosures of these documents are hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

Examples

Example 1
In vitro characterization of anti-c-*myc* DNAzymes

The efficacy of DNAzymes *in vitro* was determined by measuring the rate of RNA cleavage under multiple turnover conditions. For these experiments, a range of substrate concentrations was used such that [S] ≥ 10-fold excess over [E] which was fixed at 200 pM. The DNAzyme and a ^{32}P-labeled synthetic RNA substrate were pre-equilibrated separately for 10 minutes at 37°C in 50 mM Tris.HCl, pH 7.5, 10 mM $MgCl_2$ and 0.01% SDS. At time zero, the reaction was initiated by mixing the DNAzyme and substrate together. The reaction progress was then followed by the analysis of aliquots taken sequentially at various time points and quenched in 90% formamide, 20 mM EDTA and loading dye. The product fragments and unreacted substrate in these samples were resolved by electrophoresis on a 16% denaturing polyacrylamide gel. The extent of reaction at each time point was determined by densitometry of the gel image produced through a PhosphorImager (Molecular Dynamics). The values for k_{obs} (derived from the slopes of these time course experiments) was used to generate a line of best fit in a modified Eadie-Hofstee plot (k_{obs} vs. $k_{obs}/[S]$). In this way, the values for K_m and k_{cat} are given as the negative slope of the regression line and the y intercept, respectively.

Multiple turnover kinetics were used to examine the efficiency of DNAzyme-catalyzed cleavage of a short synthetic c-*myc* RNA sequence *in vitro* (Figure 4). Three modified DNAzymes and their unmodified controls with symmetrical 7, 8 and 9 base pair substrate-binding arms

were incubated with an excess of the ^{32}P-labeled synthetic c-*myc* RNA. From the values for k_{obs}, the kinetic parameters K_m and k_{cat} were determined (Table 1).

The overall catalytic efficiency of each DNAzyme, as measured by the k_{cat}/K_m ratios, varies significantly between the modified and unmodified species. In the short arm DNAzymes (7+7 bp), the inclusion of an inverted base modification produced a 3-fold decrease in the k_{cat}/K_m. In contrast to this negative effect on the cleavage activity, the relative efficiency of the long (9+9 bp) arm version was enhanced 10-fold by the presence of an inverted base modification. The intermediate length (8+8 bp) binding arm DNAzyme was the least effected by modification, showing a 2-fold increase in the value of k_{cat}/K_m. The effect of the 3' inverted terminal base was therefore different depending on the length of the substrate-binding arms. In the short (7+7 bp) arm DNAzyme, the modification was found to be detrimental to the catalytic efficiency. However, in the long (9+9 bp) arm molecule, it actually improved catalytic activity. The unmodified DNAzyme activity was optimal with 8 bp substrate binding arms. In the short (7 bp) arm DNAzyme, the overall efficiency was lower due mainly to a higher K_m (3.4-23 nM). In DNAzymes having arms longer than 8 bp (i.e., 9 bp), the overall efficiency was diminished as a result of both a relative rise in K_m (3.4-7 nM) and a fall in the k_{cat} (0.11-0.06 min^{-1}).

Thus, for c-*myc* mRNA-cleaving DNAzymes, optimal cleavage efficiency in the unmodified versions was observed with 8 bp arms. Both the 7 and 9 bp versions of the unmodified c-*myc* DNAzyme had lower overall

efficiencies according to their respective values for k_{cat}/K_m.

The kinetic profile of these three different size c-*myc*-cleaving molecules was altered considerably by the inclusion of a 3'-terminal nucleotide inversion. The influence of this DNA modification on the kinetics of c-*myc* RNA cleavage was particularly apparent in the short 7 bp arm DNAzyme. This molecule was substantially less efficient in terms of its value for k_{cat}/K_m compared to the unmodified version. This reduction in catalytic efficiency, however was recovered and even enhanced by the addition another two nucleotides in the 8 bp modified version. This indicated that the reduction of activity in the short DNAzyme was due to some disturbance of DNA/RNA interactions (caused by the nucleotide inversion), which could be recovered by increasing the arm lengths to 8 bp. Another slight improvement in catalytic efficiency was found by further increasing the arm lengths of the modified DNAzyme to 9 bps. This was in contrast to the situation in the unmodified DNAzyme that demonstrated a sharp decline in activity is observed when increasing arm length from 8 bp to 9 bp.

These results demonstrate that 8 bp is the optimal arm length for c-*myc* RNA cleavage by the unmodified DNAzyme. An arm length of 9 bp appears to provide the optimal catalytic cleavage activity in 3'-inverted DNAzymes. The decline in catalytic efficiency seen in the unmodified DNAzyme with 9 bp arms partially reflects a reduction in enzyme turnover rate apparent as a lower value for k_{cat}. This lower turnover rate is probably a result of the DNAzyme's increased affinity for the reaction product, which affinity in turn slows down product dissociation. This reduction of activity

was avoided in the DNA modified by terminal base inversion, possibly as a result of destabilization of the enzyme-product interactions.

Table 1

Kinetics of c-*myc*-cleaving DNAzymes

DNAzyme	Arm length	Modificat'n	K_{cat} (min^{-1})	K_m (nM)	K_{cat}/K_m ($pM^{-1} \cdot min^{-1}$)
Rs-1	7+7	None	0.25	23	10.8
Rs-2	7+7	3'inversion	0.16	50	3.2
Rs-3	8+8	none	0.11	3.4	32
Rs-4	8+8	3'inversion	0.24	4	60
Rs-5	9+9	None	0.06	7	8.6
Rs-6	9+9	3'inversion	0.26	4	65

The kinetics of c-*myc* RNA cleavage were analyzed for three different length DNAzymes (both modified and unmodified) all targeting the start codon. Reactions were performed under multiple turnover conditions with at least a 10-fold excess of substrate in the presence 10 mM $MgCl_2$ and 50 mM Tris.HCl, pH 7.5.

Example 2

In vitro cleavage of full-length c-*myc* mRNA

A full-length c-*myc* mRNA was used to further test DNAzymes' ability to cleave various forms of c-*myc* mRNA under simulated physiological conditions (10 mM $MgCl_2$, pH7.5, 37°C). Cleavage reactions were performed under single turnover conditions by using 10 nM of long substrate (c-*myc* mRNA) and 50 nM of DNAzymes.

Figure 5 shows that all the DNAzymes effectively cleave c-*myc* mRNA with a cleavage rate of 20 to 50%. As expected, the DNAzymes with longer arms cleave substrates more efficiently. A 3'-inverted base modification decreases the cleavage efficiency of the 7+7 arm DNAzyme, but increases the cleavage efficiency of the 9+9 arm DNAzyme. Interestingly, there was no difference in DNAzyme cleavage efficiency between preheated and non-preheated DNAzymes. This result indicates that the accessibility of the cleavage site within the c-*myc* mRNA is not affected by mRNA secondary structure.

Example 3
Chemical modification and stability of DNAzymes

The following method assays DNAzyme stability in 100% human AB serum. Briefly, 150 µM unlabeled DNAzyme was incubated in 100 µl 100% human serum at 37°C, and duplicate samples of 5 µl were removed at time points of 0, 2, 8, 24, 48 and 72 hours. Immediately upon sampling, 295 µl TE (10 mM Tris.Cl, pH 7.5, 1 mM EDTA) was added to the 5µl aliquot, and phenol/chloroform extraction was performed. All the samples from each time point were end-labeled with γ-^{32}P-ATP and run directly on 16% PAGE gels without further purification or precipitation, thus showing all intact DNAzymes and degradation products. Results show that a 3'-3' inversion at the 3' end significantly improved DNAzyme stability in human serum ($t_{1/2}$ = 20 hours), while unmodified DNAzyme exhibited a half-life of < 2 hours (Figure 6).

Example 4
DNAzyme-mediated inhibition of SMC proliferation

Anti-c-*myc* DNAzyme activity was tested in vascular SV40LT (Simian Virus 40 large T antigen) smooth muscle cells (Simons). After growth arrest in 0.5% FBS-DMEM, SMC's were released from G_0 by addition of 10% FBS-DMEM. Cells were simultaneously exposed to DNAzyme or control oligonuceotide (i.e., the 9/9 arm DNAzyme with an inverted catalytic core sequence) delivered by DOTAP. DNAzyme growth-inhibitory ability was measured at 72 hours after delivery. The data for different DNAzymes shown in Figure 7 reveal a range of between 30% to 80% decrease in SMC numbers, while no decrease was observed using the control. Based on these assay results, the activity of the most effective molecule, Rs-6 (9/9 arms with 3' inverted base) was examined further in a dose-response assay (Figure 8). Compared with the control, Rs-6 significantly inhibits SMC growth at concentrations of as low as 50 nM.

Example 5
Effect of anti-c-*myc* DNAzyme on SMC cell cycle

The impact of DNAzymes on SMC proliferation was also assessed using two independent techniques, i.e., DNA cell-cycle analysis and the determination of mitotic index. DNA histograms were generated at 72 hours after serum stimulation. After this 72-hour interval, 74% of unstimulated cells remained in G_0/G_1, as compared with only 65% of stimulated cells. However, with the addition of the DNAzyme Rs-6, the proportion of stimulated cells remaining in G_0/G_1 phase increased to 71%. In contrast, the inactivated DNAzyme control (Rs-8) had no effect on the SMC cycle. These results were confirmed by quantifying the mitotic indices (i.e. the number of mitoses per 1000 cells, as determined

microscopically) of SMC populations 72 hours after stimulation. Data are shown in Table 2.

Table 2

Effect of Anti-c-*myc* DNAzyme on Serum-Stimulated Smooth Muscle Cell Proliferation

	G0/G1 (%)	S (%)	G2/M (%)	Mitotic Index (%)
Unstimulated	73.66	8.56	13.39	0.5
DOTAP	65.24	12.59	16.62	1.9
Rs-6	70.81	9.93	14.12	0.3
Rs-8 (Control)	67.81	12.33	15.19	2.2

Example 6
Expression of c-*myc* protein in DNAzyme-transfected SMC's

In order to demonstrate efficacy of anti-c-*myc* DNAzymes at the molecular level, expression of c-*myc* protein in DNAzyme-treated SMC's was assayed using immunoprecipitation. Briefly, SMC's were arrested in serum-free medium for 72 hours followed by incubation in met-free medium (containing 5% dialyzed fetal calf serum) for 1 hour at 37°C. After removing the medium, the cells were replaced with met-free medium containing 5% dialyzed fetal calf serum, 100 mCi/ml ^{35}S-Met and 5 mM DNAzyme, and incubated for an additional 2 hours. The cell lysates were prepared using the protocol as described, and c-*myc* protein was detected using agarose-conjugated anti-c-*myc* antibody. As shown in

Figure 9, treatment of SMC's with anti-c-*myc* DNAzyme markedly inhibited the synthesis of c-*myc* protein, as determined by immunoprecipitation of metabolically labeled material. SMC incubation with control oligonucleotide (Rs-8) had no effect on c-*myc* expression.

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U.S. Serial No. 60/091,217, filed June 30, 1998.

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What is claimed is:

1. A DNAzyme which specifically cleaves c-*myc* mRNA, comprising
   (a) a catalytic domain that has the nucleotide sequence GGCTAGCTACAACGA and cleaves mRNA at any purine:pyrimidine cleavage site at which it is directed,
   (b) a binding domain contiguous with the 5′ end of the catalytic domain, and
   (c) another binding domain contiguous with the 3′ end of the catalytic domain,
   wherein the binding domains are complementary to, and therefore hybridize with, the two regions immediately flanking the purine residue of the cleavage site within the c-*myc* mRNA, respectively, at which DNAzyme-catalyzed cleavage is desired, and wherein each binding domain is at least six nucleotides in length, and both binding domains have a combined total length of at least 14 nucleotides.

2. The DNAzyme of claim 1, wherein each binding domain is nine nucleotides in length.

3. The DNAzyme of claim 1, wherein the 3′-end nucleotide residue is inverted in the binding domain contiguous with the 3′ end of the catalytic domain.

4. The DNAzyme of claim 1 having the sequence TGAGGGGCAGGCTAGCTACAACGACGTCGTGAC.

5. The DNAzyme of claim 1, wherein cleavage site within the c-*myc* mRNA is purine:uracyl.

6. The DNAzyme of claim 1, wherein the cleavage site within the c-*myc* mRNA is located in a region selected from the group consisting of the translation initiation site, a splice recognition site, the 5′ untranslated region, and the 3′ untranslated region.

7. A pharmaceutical composition for inhibiting the onset of restenosis, which comprises the DNAzyme of claim 1 and a pharmaceutically acceptable carrier suitable for topical administration.

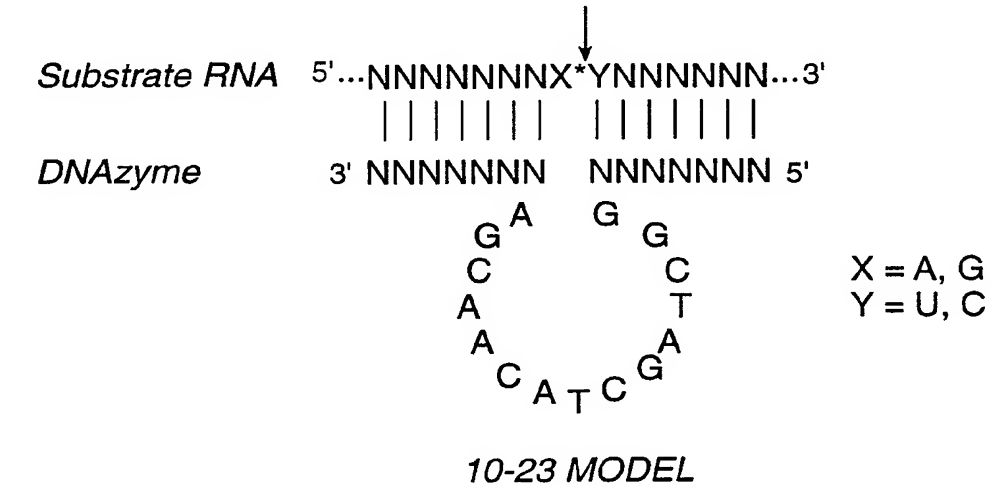
8. The pharmaceutical composition of claim 7, wherein the pharmaceutically acceptable carrier is selected from the group consisting of a liposome and a biodegradable polymer.

9. An angioplastic stent for inhibiting the onset of restenosis, which comprises an agioplastic stent operably coated with a prophylactically effective dose of the pharmaceutical composition of claim 7.

10. A method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a prophylactically effective dose of the pharmaceutical composition of claim 7 to the subject at around the time of the angioplasty.

11. A method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering the angioplastic stent of claim 9 to the subject at the time of the angioplasty.

FIG. 1



10-23 MODEL

FIG. 2

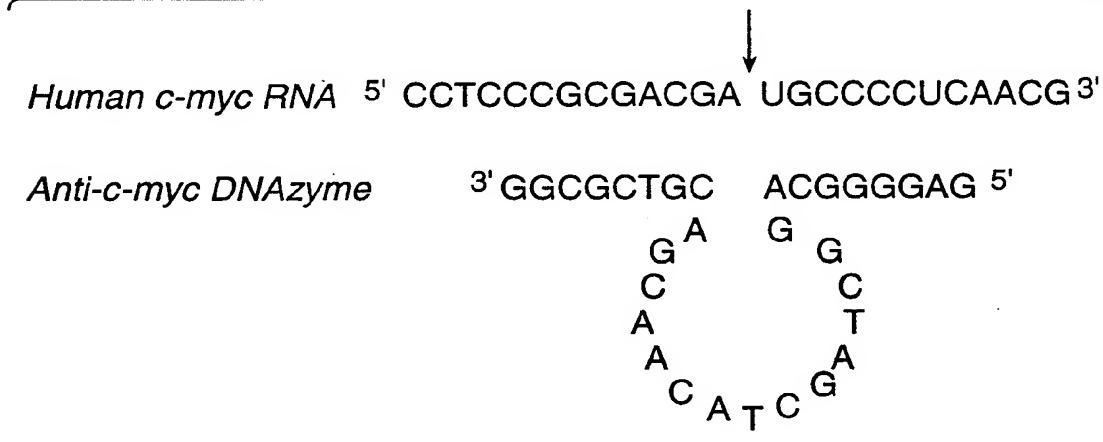


FIG. 3

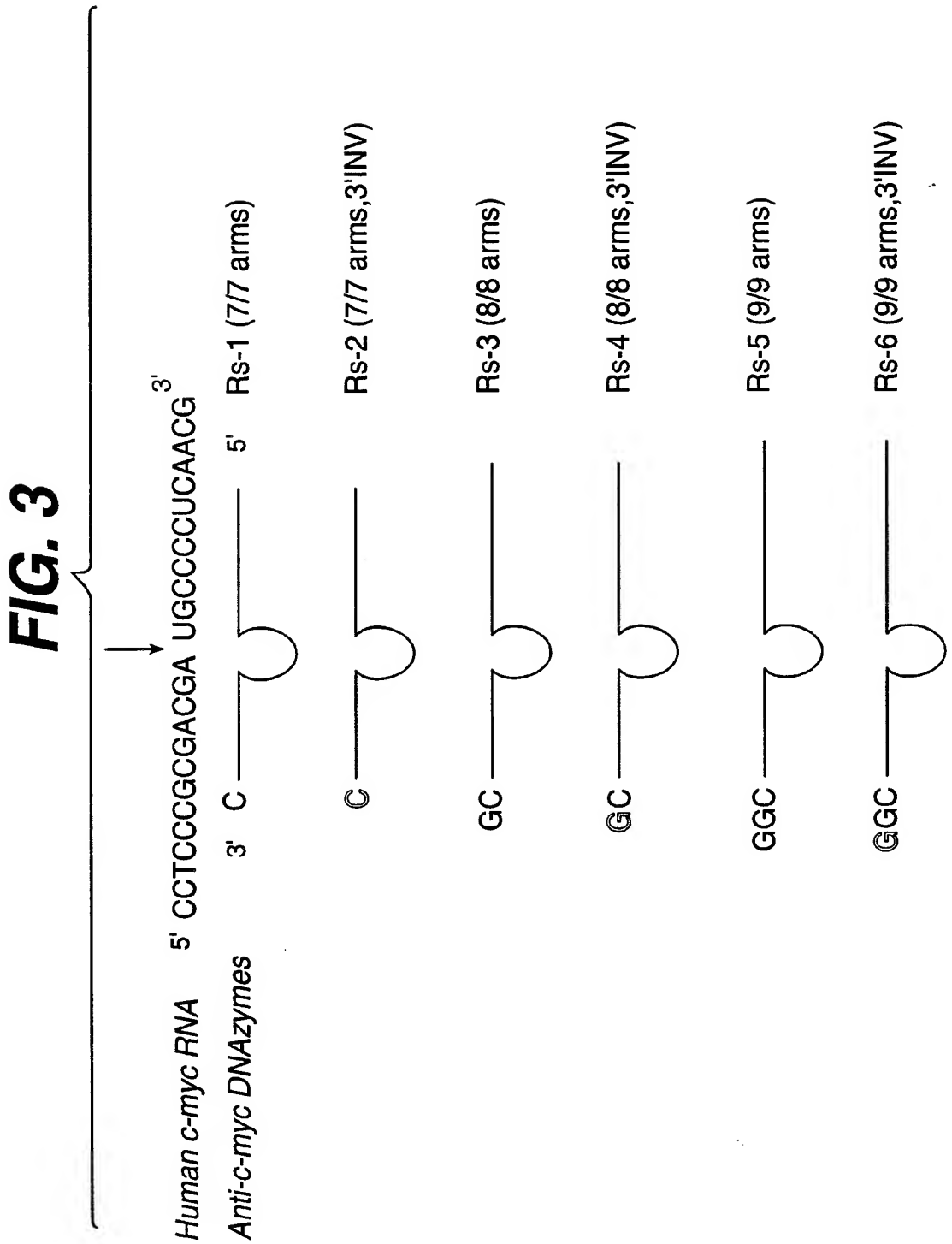

Human c-myc RNA 5' CCTCCCGCGACGA UGCCCCUCAACG 3'
Anti-c-myc DNAzymes
3' C 5' Rs-1 (7/7 arms)
C Rs-2 (7/7 arms,3'INV)
GC Rs-3 (8/8 arms)
GC Rs-4 (8/8 arms,3'INV)
GGC Rs-5 (9/9 arms)
GGC Rs-6 (9/9 arms,3'INV)

FIG. 4A

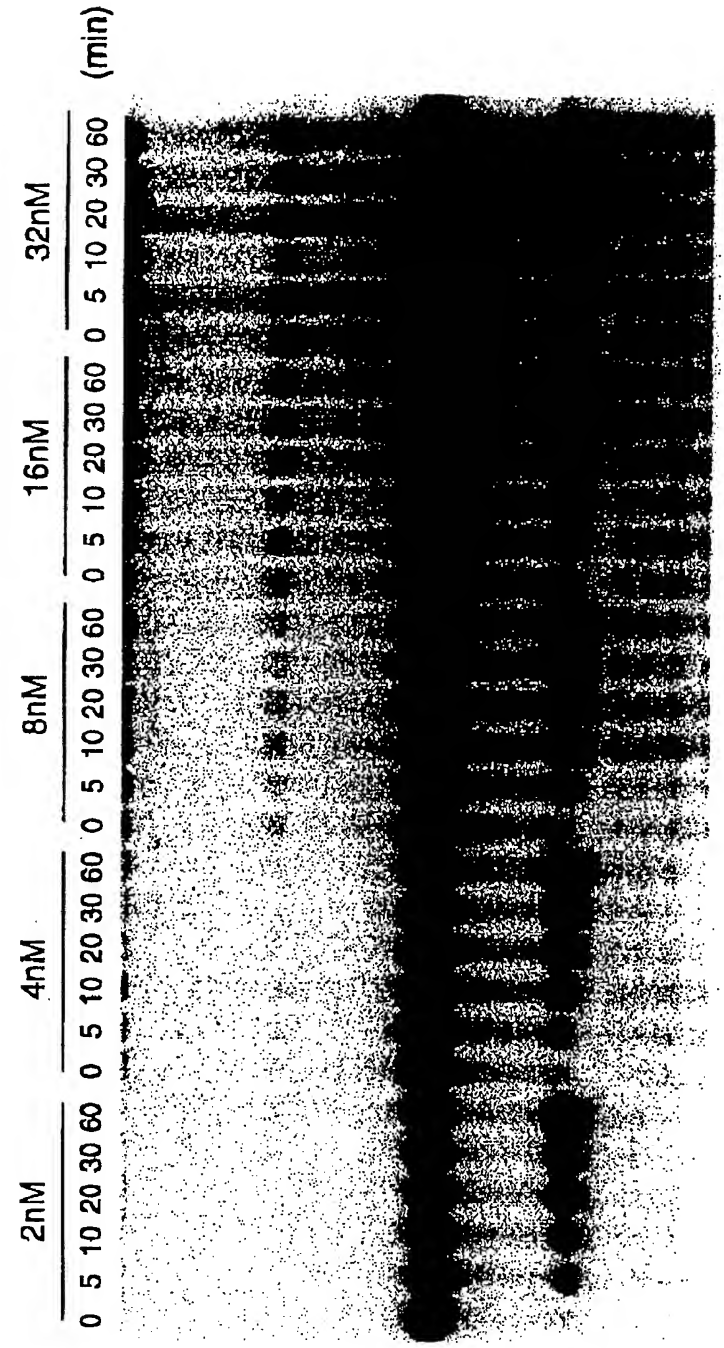

2nM
4nM
8nM
16nM
32nM
0 5 10 20 30 60 0 5 10 20 30 60 0 5 10 20 30 60 0 5 10 20 30 60 0 5 10 20 30 60 (min)

FIG. 4B

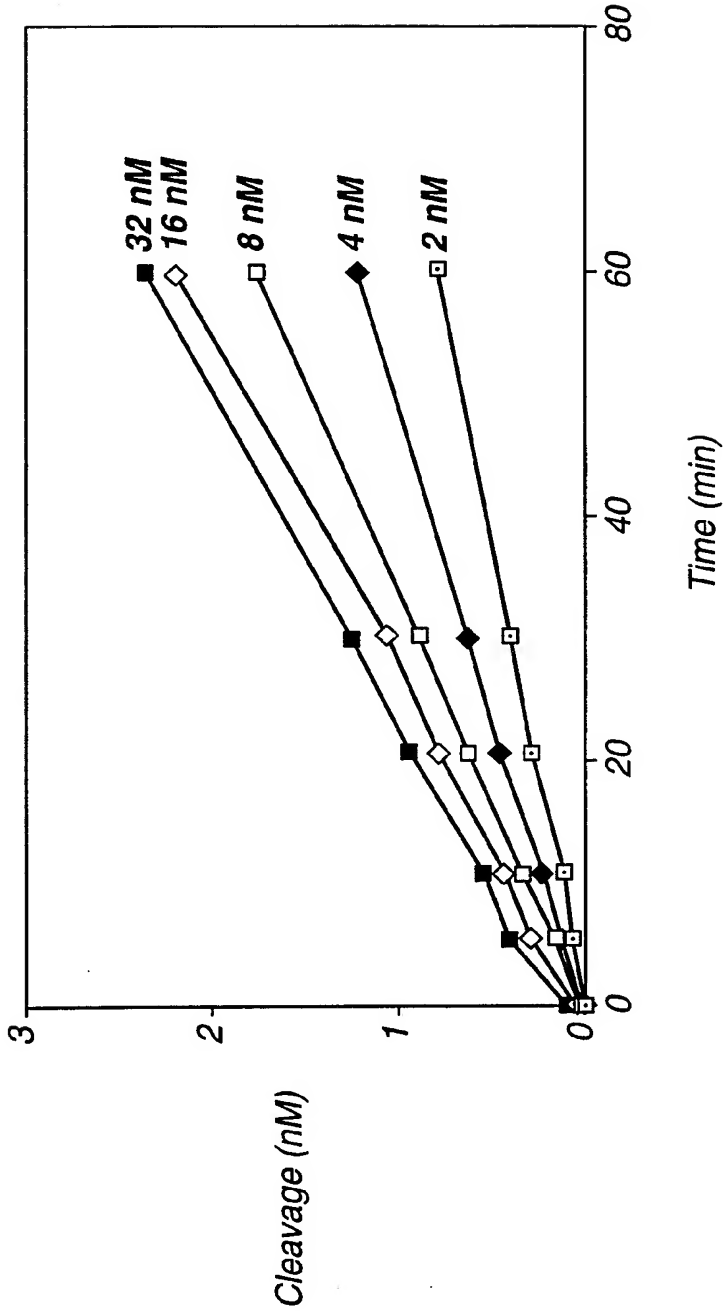


FIG. 5

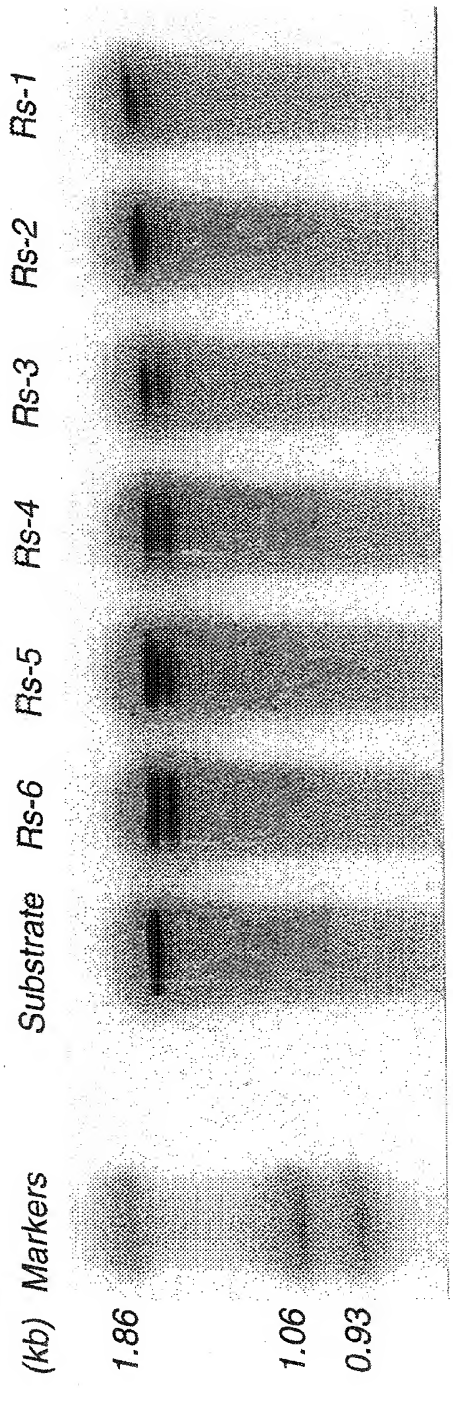


FIG. 6

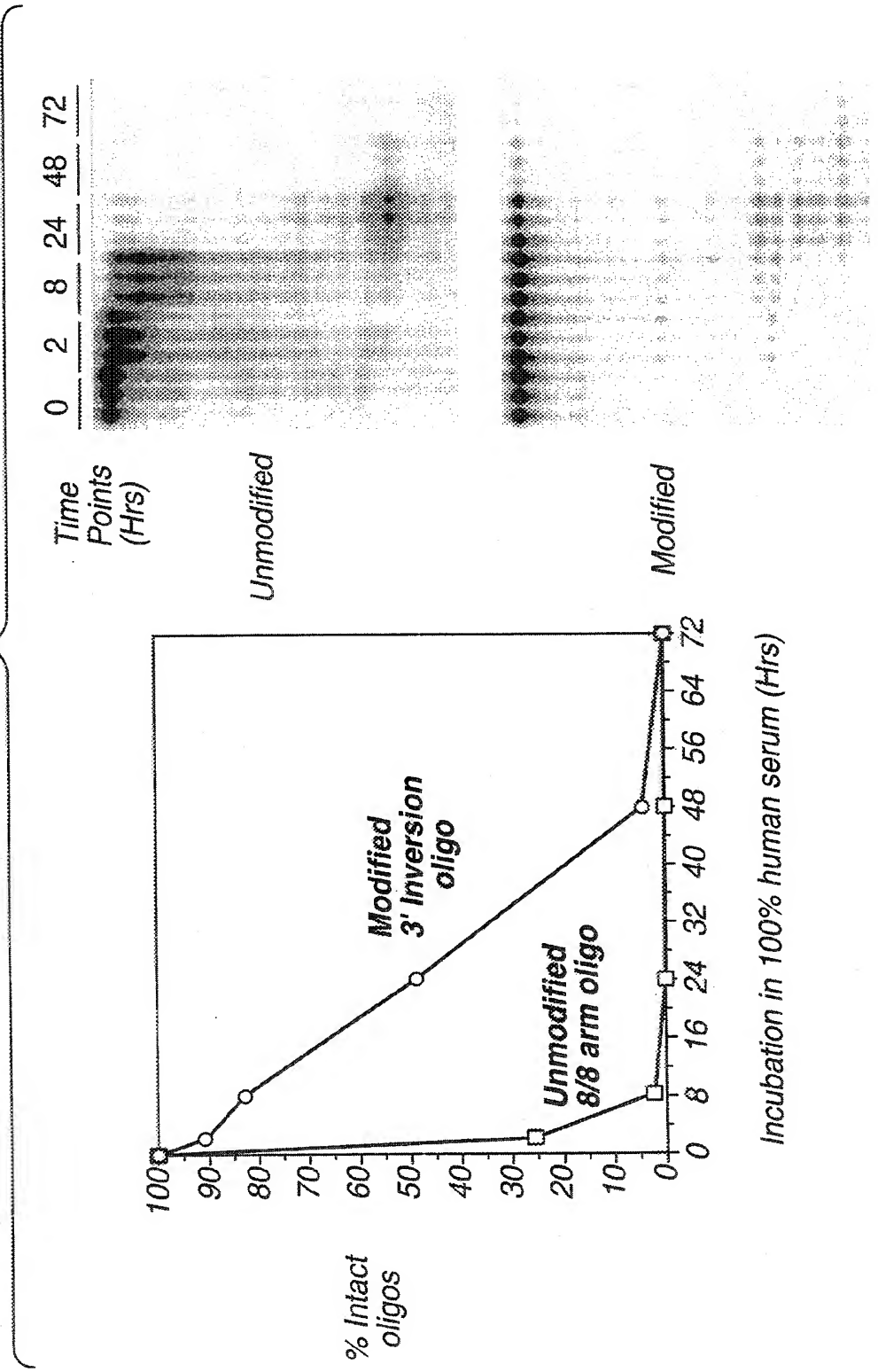

Time Points (Hrs)
0 2 8 24 48 72
Unmodified
Modified
% Intact oligos
100 90 80 70 60 50 40 30 20 10 0
Modified 3' Inversion oligo
Unmodified 8/8 arm oligo
0 8 16 24 32 40 48 56 64 72
Incubation in 100% human serum (Hrs)

FIG. 7

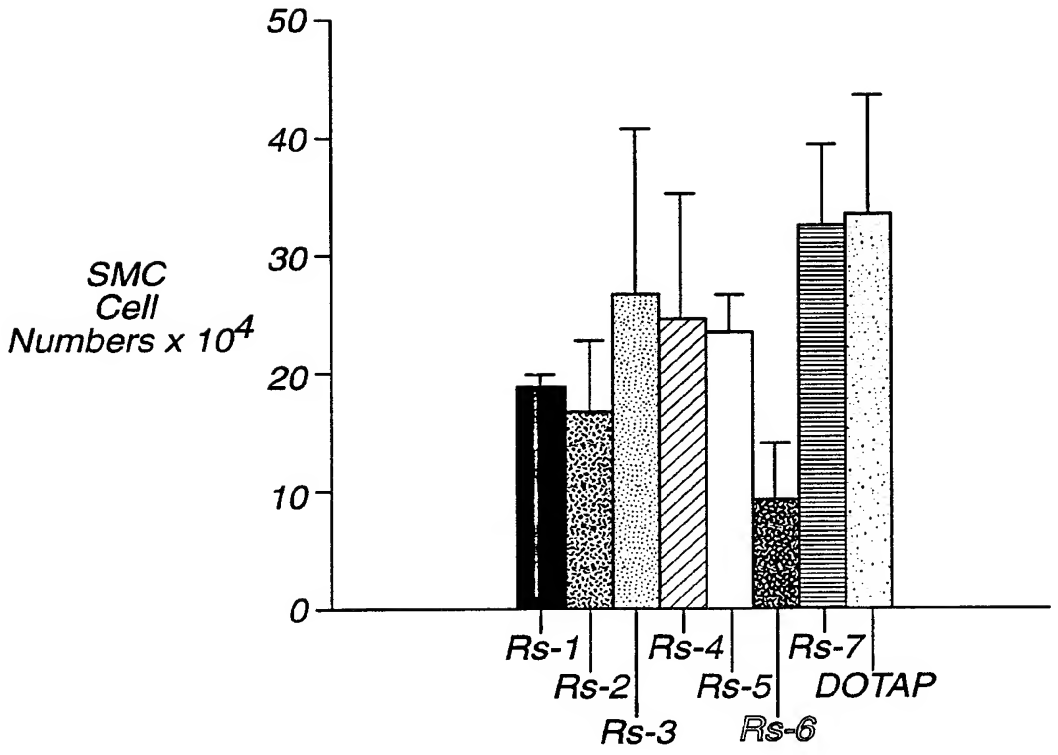

SMC
Cell
Numbers x 10^4
50
40
30
20
10
0
Rs-1
Rs-2
Rs-3
Rs-4
Rs-5
Rs-6
Rs-7
DOTAP

FIG. 8

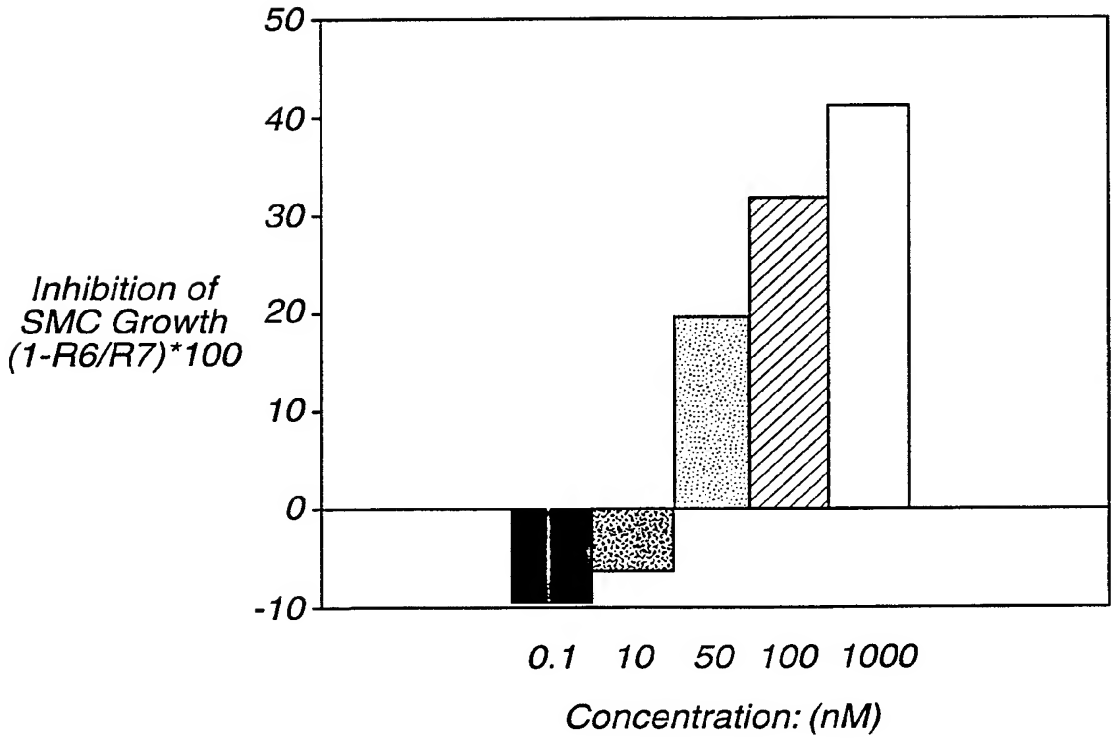

50
40
30
20
10
0
-10
Inhibition of SMC Growth (1-R6/R7)*100
0.1
10
50
100
1000
Concentration: (nM)

FIG. 9

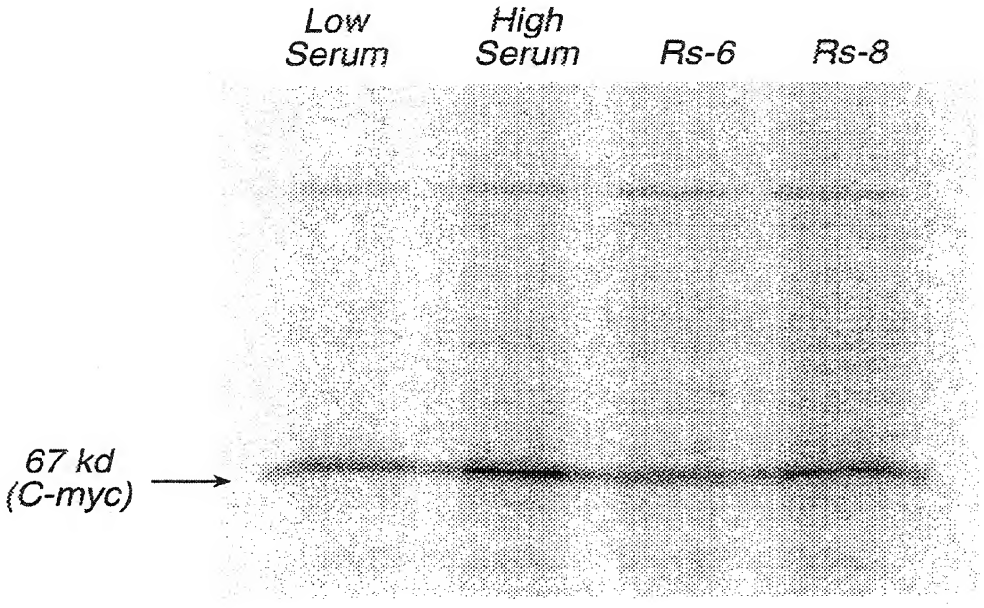


FIG. 10A

c-myc Genomic DNA Sequence

```
   1 ttctcgtgtg gagggcagct gttccgcctg gcgtagattt atactcacatg gataaggtaa
  61 cggtttgtca aacagtactg ctacggagga gcagcagaga aagggagagg gtttgagagg
 121 gagcgaaaag aaaatggtag gcgcgcgtag ttaattcaat gcggctctct tactctgttt
 181 acatcctaga gctagagtgc tcggctgccc ggctgagtct cctcccccacc ttcccccaccc
 241 tccccaccct cccataagc gccctcccgg gttcccaaag cagagggcgt gggggaaaag
 301 aaaaaagatc ctctctcgct aactctccgc ccaccggccc tttataatgc gagggtctgg
 361 acggctgagg acccccgagc tgtgctgctc gcggccgcca ccgccgggcc ccggccgtcc
 421 ctggctcccc tcctgcctcg agaagggcag ggcttctcag aggcttggcg ggaaaaagaa
 481 cggagggagg gatcgcgctg agtataaaag ccggttttcg gggcttttatc taactcgctg
 541 tagtaattcc agcgagaggc agagggagcg agcgggcggc cggctagggt ggaagagccg
 601 ggcgagcaga gctgcgctgc gggcgtcctg ggaagggaga tccggagcga atagggggct
 661 tcgcctctgg cccagccctc ccgctgatcc cccagccagc ggtccgcaac cctgccgca
 721 tccacgaaac tttgcccata gcagcgggcg ggcactttac gactggaact tacaacaccc
 781 gagcaaggac gcgactctcc gacgcggggga ggctattctg cccatttggg gacacttccc
 841 cgccgctgcc aggacaccgc ttctctgaaa ggctctcctt gcagctgctt agacgctgga
 901 ttttttcggg gtagtggaaa accaggtaag caccgaagtc cacttgcctt ttaatttatt
 961 ttttatcac tttaatgctg agatgagtcg aatgcctaat ctttctctccc attcctgcgc
1021 tattgacact tttctcagag tagttctagg taactgtggggc tggggtgtgggg gtaatccaga
1081 actggatcgg ggtaaagtgc attgcaagat gggagaggag aagggcagagg gaaacgggaa
1141 tggttttaa gactaccctt tcgagatttc tgccttatga atatattcac gctgactccc
1201 ggccgggtcg gacattcctg ctttattgtg ttaattgctc tctgggtttt gggggctggg
1261 ggttgtttgg cggtggcaag aaagccctg catcctgagc tccttggagt acggaccgca
1321 ttatcgcctg tgtgagccag atcgctccgc agccgctgac ttgtcccgt ctccgggagg
1381 gcatttaaat ttcggctcac cgcatttctg acagccggag acggacactg cggcgctcc
```

FIG. 10B

```
1441 cgcccgcctg tccccgcggc gattccaacc cgccctgatc cttttaataa attccgattt
1501 ggctttttaa aaagcaataa tacaatttaa aacctgggtc tctagaggtg ttaggacgtg
1561 gtgttgggta ggcgcaggca ggggaaaagg gaggcgagga tgtgtccgat tctccttgga
1621 atcgttgact tggaaaaacc agggcgaatc tccgcaccca gccctgactc ccctgccgcg
1681 gccgccctcg ggtgtcctcg cgcccgagat gcggaggaac tgcgaggagc ggggctctgg
1741 gcggttccag aacagctgct acccttggtg gggtggctcc ggggcaggta tcgcagcggg
1801 gtctctggcg cagttgcatc tccgtattga gtgcgaaggg aggtcccccc attattattt
1861 gacacccccc ttgtatttat ggaggggtgt taaagcccgc ggctgagctc gccactccag
1921 ccggcgagag aaagaagaaa agctggcaaa aggagtgttg gacgggggcg gtactggggg
1981 tggggacggg ggcggtggag agggaaggtt gggaggggct gcggtgccgg cgggggtagg
2041 agagcggcta gggcgcgagt gggaacagcc gcagcggagg ggccccggcg cggagcgggg
2101 ttcacgcagc cgctagcgcc caggcgcctc tcgccttctc cttcaggtgg cgcaaaactt
2161 tgtgccttgg attttggcaa attgttttcc tcaccgccac ctcccgcggc ttcttaaggg
2221 cgccagggcc gatttcgatt cctctgccgc tgcggggccg actcccgggg ctttgcgctc
2281 cgggctcccg ggggagcggg ggctcggcgg gcaccaagcc gctggttcac taagtgcgtc
2341 tccgagatag caggggactg tccaaagggg gtgaaagggt gctcccttta ttccccccacc
2401 aagaccaccc agccgcttta ggggatagct ctgcaagggg agaggttcgg gactgtggcg
2461 cgcactgcgc gctgcgccag gtttccgcac caagacccct ttaactcaag actgcctccc
2521 gctttgtgtg ccccgctcca gcagcctccc gcgacgatgc ccctcaacgt tagcttcacc
2581 aacaggaact atgacctcga ctacgactcg gtgcagccgt atttctactg cgacgaggag
2641 gagaacttct accagcagca gcagcagagc gagctgcagc ccccggcgcc cagcgaggat
2701 atctggaaga aattcgagct gctgcccacc ccgcccctgt cccctagccg ccgctccggg
2761 ctctgctcgc cctcctacgt tgcggtcaca cccttctccc ttcggggaga caacgacggc
2821 ggtggcggga gcttctccac ggccgaccag ctggagatgg tgaccgagct gctgggagga
```

FIG. 10C

```
2881 gacatggtga accagagttt catctgcgac ccggacgacg agaccttcat caaaaacatc
2941 atcatccagg actgtatgtg gagcggcttc tcggccgccg ccaagctcgt ctcagagaag
3001 ctggcctcct accaggctgc gcgcaaagac agcggcagcc cgaacccccgc ccgcggccac
3061 agcgtctgct ccacctccag cttgtacctg caggatctga gcgccgccgc ctcagagtgc
3121 atcgaccccct cggtggtctt cccctaccct ctcaacgaca gcagctcgcc caagtcctgc
3181 gcctcgcaag actccagcgc cttctctccg tcctcggatt ctctgctctc ctcgacggag
3241 tcctcccccgc agggcagccc cgagcccctg gtgctccatg aggagacacc gcccaccacc
3301 aacagcgact ctggtaagcg aagc
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 99/01484

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 9/16, A61K 38/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, CA: KEYWORDS (KW) See the electronic data base box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE, EMBL, GENBANK, DDBJ, PDB and Dgene (Derwent database)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DNA enzyme# or DNAzyme# or deoxyribozyme# or (catalytic DNA and myc). Nucleic acid sequences: ggctagctacaacga, and tgaggggcaggctagctacaacgacgtcgtgac.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X P..Y	WO 98/49346 (THE SCRIPPS RESEARCH INSTITUTE) 5 November 1998 priority date 29 April 1997. See the whole document, especially pages 95-98, sequence id no. 85 and figures 8-10	1-6. 7-11.
P, X	Sun L-Q *et al* "Suppression of Smooth Muscle Cell proliferation by a c-myc RNA-cleaving deoxyribozyme" The Journal of Biological Chemistry vol 274 June 11 1999, pp. 17236-17241. See the whole document especially pages 17238 -17241	1-11.
P, X	Cairns M. J. *et al* "Target site selection for an RNA-cleaving catalytic DNA" Nature Biotechnology vol 17, May 1999 pp 480-486. See the whole document.	1-11.

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 December 1999	15 DEC 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **J.H. CHAN** Telephone No.: (02) 6283 2340

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 99/01484

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	WO 96/17086 (THE SCRIPPS RESEARCH INSTITUTE) 6 June 1996. See the whole document, especially pages 11, 12, 51-54, sequence id no. 85 and figures 8 and 9	1-11.
X Y	Santoro S W and Joyce G F "A general purpose RNA-cleaving DNA enzyme" Proc Natl Acad Sci USA vol **94** pp 4262-4266 April 1997. See the whole document especially pp 4264-6 and figure 2.	1-6. 7-11.
Y	Genbank accession no. J00120 publication date 25 July 1994.	1-6.
Y	Bernard O *et al* "Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumours" EMBO J 1983; **2**(12);2375-2383. See the whole document, especially the sequences therein.	1-6.
P, X P, Y	Santoro S W and Joyce G F "Mechanism and utility of an RNA-cleaving DNA enzyme" Biochemistry 1998 Sept 22, **37**, 13330-42 See the whole document especially pp 13331, 13337-41 and figure 1	1-6. 7-11.
P, X	WO 99/50452 (JOHNSON & JOHNSON RESEARCH PTY LIMITED) 7 October 1999. See the whole document, especially pp 17-25.	1-11.
P,Y	Warashina M *et al* "Extremely high and specific activity of DNA enzymes in cells with a Philadelphia chromosome" Chemistry & Biology 1999 vol 6 pp 237-250. See the whole document especially figures 2. 4 and page 247.	1-11.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB 99/01484

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	98/49346	AU	72675/98				
WO	96/17086	AU	45950/96	BR	9510003	CA	2205382
		CN	1173207	EP	792375	FI	972333
		HU	77576	NO	972483	US	5807718
WO	99/50452	AU	35303/99				

END OF ANNEX